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Comparison of System Design and Reproducibility to Estimate Bioconcentration of Di-n-Hexylphthalate By 'Daphnia magna'

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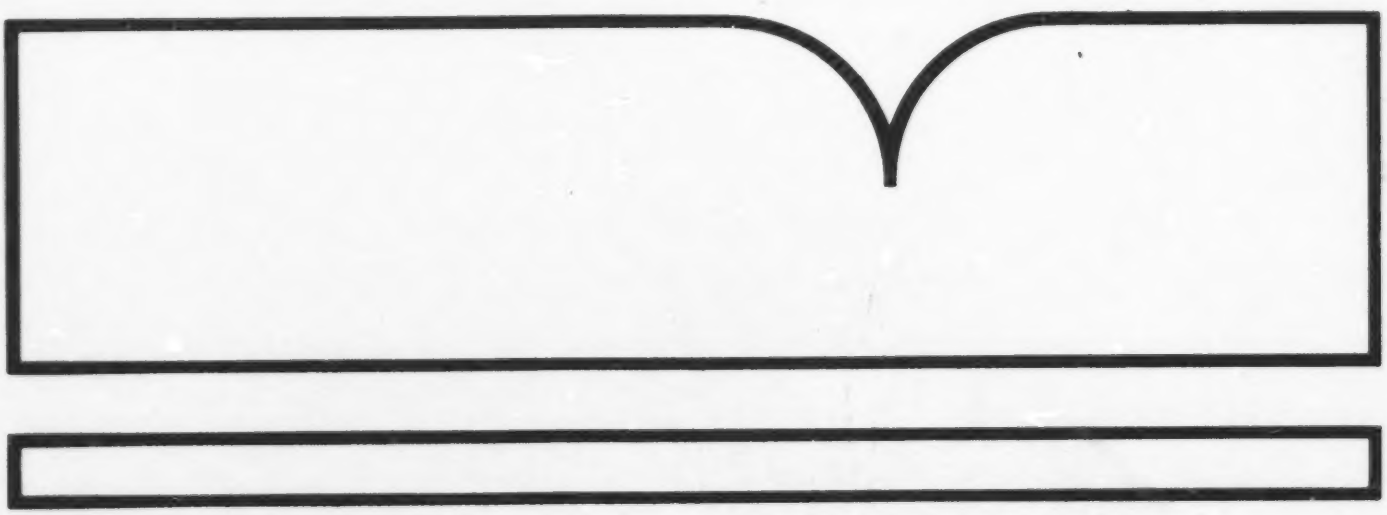

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COMPARISON OF SYSTEM DESIGN AND REPRODUCIBILITY TO ESTIMATE BIOCONCENTRATION OF DI-*N*-HEXYLPHTHALATE BY *DAPHNIA MAGNA*

Steven P. Gloss and Gregory R. Biddinger

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Comparison of System Design and Reproducibility to Estimate Bioconcentration of Di-*n*-hexylphthalate by *Daphnia magna*

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Exposures to ^{14}C-DHP in water only (*Daphnia* fed) were conducted in a mixed model sys-

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tem wherein *Daphnia* were subjected to controlled predation by bluegill sunfish (*Lepomis macrochirus*). Results from the mixed model systems suggested that steady-state accumulation took longer than seven days to achieve. The measured bioconcentration factor (BCF) of 3250 for ^{14}C-DHP in *D. magna* was similar to values reported for other phthalate esters in microcrustaceans.

Measured BCFs for the water only (48 h), food plus water (48 h), and the mixed model system (14 days) were 1066, 1486, and 3254, respectively. Calculated BCFs, using a first-order steady-state equilibrium model were 998, 1414, and 4655, respectively, for the same three treatments. All BCFs reported are for total ^{14}C activity on a wet-weight basis.

KEY WORDS: microcosm, aquatic food chain, bioconcentration, *Daphnia magna*, *Selenastrum capricornutum*, di-*n*-hexylphthalate

The use of microcosms is one way to estimate the potential bioaccumulation of organic chemicals in aquatic organisms [*1, 2*]. Bioaccumulation estimates or bioconcentration factors (BCFs) may also be obtained by using various physical-chemical characteristics of the test chemical [*3*]. The need to screen chemicals for their potential to accumulate in tissue is integral to full hazard assessment. The relative importance of chemical uptake from the food chain versus direct uptake from water has been discussed by several authors [*3–5*].

The ultimate indication of bioaccumulation results from measurements of samples from the environment. However, these kinds of measurements are only available after a problem exists. While the logarithm of the *n*-octanol/water partition coefficient (log *P*) can be used to predict the log BCF, the only certain estimate is to conduct a laboratory bioconcentration test [*3*]. The BCFs estimated by either method may substantially underestimate the true BCF if the food chain represents an important vector of uptake. Additional models proposed to predict BCFs in fish require laboratory-derived uptake and depuration rate constants for validation which are often unavailable [*6*].

The design of laboratory microcosms to identify and quantify sources of accumulated chemicals in various organisms presents a substantial challenge to the researcher. Designs which mimic a complete ecosystem [*7*] are purported to estimate what has been called "ecological magnification" (EM) under laboratory conditions. Variously designed simple exposure systems may produce very different BCFs for the same chemical and the same organism. Brown and Thompson [*8*] used direct aqueous exposure to resolve differences in previously reported BCFs of di-2-ethylhexylphthalate (DEHP) in *Daphnia magna* ranging from 518 [*5*] to 2400 [*9*]. The higher BCF was obtained from a model ecosystem [*7*] type of exposure, which suggests that feeding might influence the degree of bioconcentration. What portion of the observed variation was attributable to system design versus other factors is not clear. The order of magnitude differences observed bring into question which system, if either, represents reality and whether there is an inherent lack of reproducibility in tests of this kind.

Most investigators replicate exposures within experiments on a regular basis and occasionally experiments are repeated to verify the observed responses [*9*].

There has been, however, relatively little attention paid to the reproducibility of experimental results for bioconcentration studies either within a laboratory or among different laboratories.

The purpose of this work was to evaluate two different approaches for estimating the residue dynamics of synthetic chemicals in simple aquatic food chains. The first, a modular food chain model proposed by Johnson [*1*], was designed to isolate the contributions of contaminant from water and trophic sources at each level in the food chain (algae-*Daphnia*-fish). The intent was to identify the pathways and their contributions to the total contaminant load of each successive trophic level. The second, a multispecies (mixed) food-chain model proposed by Isensee and Yockim [*10*], examined the residues in organisms after a synchronous and sympatric exposure. Total residue for each trophic level was obtained, but the significance of possible sources of contamination could not be identified or quantified. The only other substantial difference in the design of these systems is that of the exposure duration for *Daphnia*. Johnson [*1*] suggests that 48 h is sufficient to obtain an estimate of bioconcentration for *Daphnia*, whereas Isensee and Yockim [*10*] propose a full 28-day exposure. Therefore there is a possibility that the estimates of potential residues in *Daphnia* could be significantly different.

The objectives of this study were (1) to compare bioconcentration estimates for *Daphnia magna* from the two proposed types of food chain microcosms, (2) to examine the replication and reproducibility of microcosm-derived estimates of bioconcentration, and (3) to evaluate the significance of food versus aqueous sources of the chemical. A phthalic acid ester, di-*n*-hexylphthalate (DHP), recently reported as an environmental contaminant hypothesized to accumulate in fish via the food chain [*11*], was the chosen test chemical.

Methods and Materials

Ring-labeled ^{14}C-di-*n*-hexylphthalate, with a specific activity of 11.67 mCi/m*M* was obtained from Pathfinder Laboratories, St. Louis, Missouri. This material had a radiochemical purity of greater than 98% as determined by thin layer and liquid chromatography.

Daphnia magna specimens for the tests were obtained from static batch cultures maintained in 450-L fiberglass tanks filled with dechlorinated tap water. The daphnids were fed a combination of a Cerophyl/trout chow diet [*12*] and the algae *Selenastrum capricornutum*. The feeding levels were 0.1 mL/L and 1.25 mg/L [*13*], respectively. Algae were batch cultured in 16-L glass jars in accordance with techniques used by Miller et al [*14*] using a fortified version of the Marine Biological Laboratory medium [*15*]. Stock cultures were maintained at cell counts of approximately 1.8×10^7 cells/mL and a dry weight of 0.25 mg algae/mL.

All tests were performed in 70-L glass aquaria. The daphnids were exposed in accordance with either the single-species (modular) model described by

Johnson [*1*], which allows for isolation of contaminant sources (e.g., water, food), or the multispecies (mixed) model described by Isensee and Yockim [*10*]. Aquaria used for the mixed model exposures were modified with a glass partition (Fig. 1) that contained a stainless steel screen gateway with a mesh (0.23 mm) small enough to prevent passage of first-instar *Daphnia* [*10*]. This gateway allowed water circulation between the divisions, but also could be replaced with a larger mesh (5.0 mm) screen to allow controlled predation by bluegills (*Lepomis macrochirus*) which were present in the other half of the aquarium.

Dechlorinated tap water with a temperature of 21 ± 1.0°C, pH of 7.8 to 8.2, dissolved oxygen (DO) greater than 8.0 mg/L, and hardness of approximately 150 mg/L as $CaCO_3$ was used as the test medium. The photoperiod was a 12 h light/dark cycle with 37 to 42 lx of illumination. Water quality was monitored by analyzing for DO, pH, temperature, and hardness on a daily basis.

The contaminant (^{14}C-DHP) was dissolved in a solution of 20% *N'*,*N'*,dimethylformamide in distilled H_2O (1:4). The final concentration of the solvent in exposure medium was less than 0.01%. An automatic pipettor (Micromedic

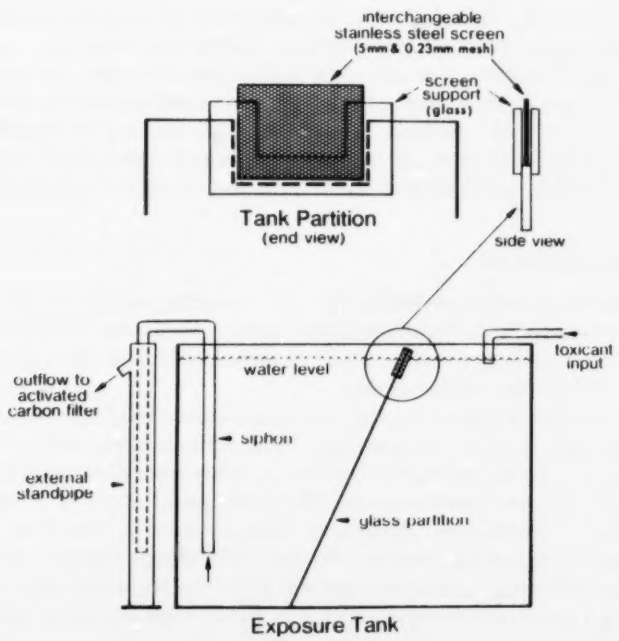

FIG. 1—*Diagram of 70-L glass aquarium with modifications for mixed model (multispecies) exposures to examine food chain transfer of chemical contaminants (after Isensee and Yockim* [10]*).*

Systems, Horesham, Pa.) was used to meter the introduction of ^{14}C-DHP into the delivery system. A solenoid-type diluter (Ace Glass, Vineland, N.J.) delivered the chemical to exposure tanks at a flow rate of 4.5 L/h. Overflow on each tank was removed by external standpipes leading to a common drain, and the effluent was filtered through activated carbon (Fig. 1). Before the introduction of test organisms, diluters were run for 48 h and calibrated to reduce differences in flow to individual aquaria.

Procedures

Daphnids exposed using the modular [*1*] approach were grouped into water only (unfed), food only, and food plus water, while the mixed species models were exposed via water only and animals were fed unlabeled algae. All treatments and controls were replicated three times per diluter; two diluter systems were run concurrently. Duplicate samples were taken from individual aquaria. The tests were a 48-h uptake period followed by a 48-h depuration for the modular method and a 28-day uptake followed by a 28-day depuration for the mixed model. The mixed model exposure was run in the same diluters immediately after the compartmental exposures were completed.

At the beginning of the tests each tank received 7 g of *D. magna* containing a mixture of sizes. The daphnids were netted with a 0.5-mm mesh nylon net, drained, weighed in a tared container, and then quickly resuspended in water before introduction to treatment tanks. All groups except the water only exposure group received 75 mg algae/day in three 25-mg aliquots at 6-h intervals. The food only and food plus water groups received algae that had been labeled with ^{14}C-DHP. To label the algae a 0.1 mg algae/mL solution containing 25 counts per minute (cpm) ^{14}C-DHP/mL or 3.25×10^{-4} μg ^{14}C-DHP/mL was placed in a rotary incubator (New Brunswick Model B16) and maintained at 21°C under continuous illumination (37 lx) at 100 rotations/min for 24 h. The algae was labeled at a rate of 0.11 μg ^{14}C-DHP/g. Before feeding, the algae was centrifuged and the supernatant discarded. The algal pellet was resuspended in water from the tank receiving the food and introduced by Pasteur pipette.

In the mixed model the screen gateways were switched to a larger mesh size for 1 h daily. This allowed daphnids of all sizes to randomly migrate into the bluegill compartment. This simulation of predation caused a reduction in *Daphnia* populations which required replenishment of the *Daphnia* stocks in some tanks on a weekly or biweekly basis.

Sampling for the modular tanks took place at 1, 3, 7, 24, and 48 h after initiating uptake and depuration, while the mixed model system was sampled at 1, 3, 7, 14, 21, and 28 days. These were the sampling intervals described by the respective authors [*1, 10*]. A 100 to 200 mg sample of *Daphnia* was removed from each tank by dip-netting, washed three times in clean water, and transferred without water to glass vials and frozen. To process, samples were thawed at

room temperature and flushed from the vial with water onto a vacuum filter for 30 s. The sample was divided in half, removed from the filter paper (Whatman #1), placed in a tared quartz-glass combustion boat, and weighed to the nearest 0.1 mg. Samples were combusted in a tissue oxidizer and the $^{14}CO_2$ evolved was trapped in scintillation cocktail (R. J. Harvey Instrument Corporation).

To estimate the ^{14}C-DHP concentrations in water, a 50-mL sample of water was taken from each tank concurrently with *Daphnia* sampling. The water was filtered (Whatman Glass fiber GF/A) and 10 mL of the filtrate was combined with 10 mL of scintillation cocktail (3a70B-Research Products International). The activity of all samples was measured in a Beckman (LS355) liquid scintillation counter until a 2% efficiency was reached or up to 10 min. The minimum concentration that was distinguishable from background was 1.31×10^{-4} μg ^{14}C-DHP/g. All values reported are in μg ^{14}C-DPH based on total activity (CPM). No analyses of metabolites were made.

Comparisons of measured differences in DHP concentration between treatment and diluters were performed by ANOVA [*16*] and Duncan's new multiple-range test [*17*].

A one-compartment model described by Hamelink [*8*]

$$(dC_a/dt) = K_1C_w - K_2C_a \tag{1}$$

where

C_a = concentration of contaminant in animal,
C_w = concentration of contaminant in water,
K_1 = uptake rate constant,
K_2 = depuration rate constant, and
t = exposure time,

was used to estimate BCF. Under conditions of constant C_w and a steady-state ($dC_a/dt = 0$) for C_a, then

$$\text{BCF} = C_a/C_w = K_1/K_2 \tag{2}$$

Estimations of K_1 and K_2 were obtained using the iterative least-squares procedure NLIN, Statistical Analysis Systems [*19*].

Results

The nominal concentration of ^{14}C-DHP in water during all exposures was 0.22 μg/L. Measured levels during the 28-day uptake experiment in all treatments and replicates where ^{14}C-DHP was added via the water averaged 0.21 μg/L (±0.025 μg/L standard deviation, $n = 36$).

No ^{14}C-DHP was detectable in any *D. magna* samples from treatments where ^{14}C-DHP was introduced only as food (algae containing 0.11 μg ^{14}C-DHP/g) or

in controls. However, ^{14}C-DHP uptake was evident in treatments where ^{14}C-DHP was introduced via the water (water only unfed and mixed model) as well as when ^{14}C-DHP was introduced in both food and water (Figs. 2 and 3). Residue levels in *Daphnia* continued to increase through the 48-h uptake period in the modular tanks. *Daphnia* stocks did not sustain themselves in the mixed model system for longer than 14 days. Each of the mixed model tanks required restocking after sampling at from 7 to 14 day intervals to maintain sufficient *Daphnia* for sampling purposes.

The maximum level of ^{14}C-DHP in *Daphnia* occurred on Day 14 in a single replicate of the mixed model system where the mean residue reached 0.716 μg/g. This represents a BCF of 3250 using the nominal water level of ^{14}C-DHP. This replicate was the only one of six in which the *Daphnia* population did not have to be supplemented. Four replicates of the mixed model, two in each diluter, maintained adequate *Daphnia* levels through seven days. Mean residues in these four tanks ranged from 0.286 to 0.627 μg ^{14}C-DHP/g.

The highest mean level of ^{14}C-DHP in *Daphnia* from the modular system was 0.34 μg/g and ranged from 0.27 to 0.43 μg/g. These highest levels occurred in the food plus water exposures and were significantly greater ($P < 0.05$) than the residues resulting from exposure via water only. Food plus water values were significantly different than water only at 48 h in both diluters; however, neither treatment was different from its duplicate in the other diluter. The maximum BCF measured in the modular model was 1545, about one half that measured on Day 14 in the mixed model. The average accumulated ^{14}C-DHP in

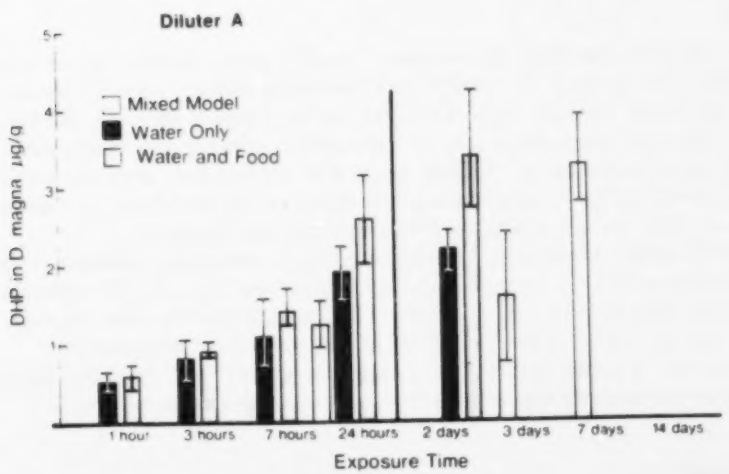

FIG. 2—*Uptake of di-n-hexylphthalate by* D. magna *exposed in modular or mixed model microcosms in diluter A (vertical bars represent range of replicate values).*

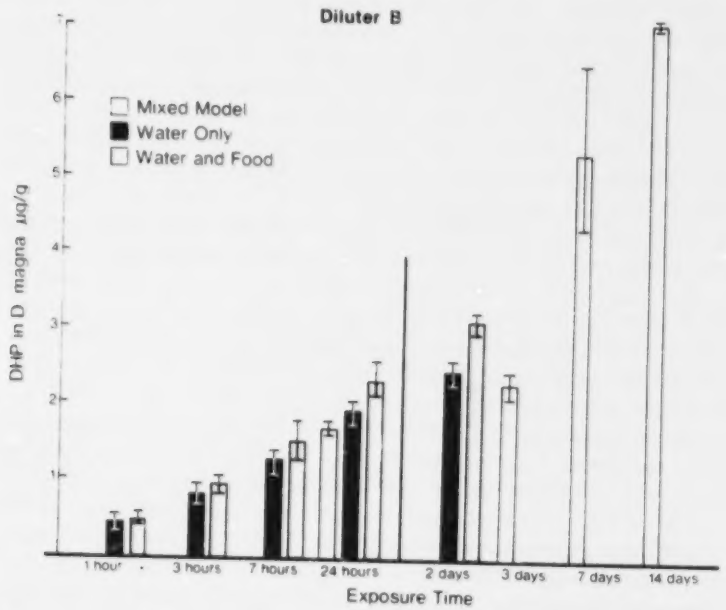

FIG. 3—*Uptake of di-n-hexylphthalate by* D. magna *exposed in modular or mixed model microcosms in diluter B (vertical bars represent range of replicate values).*

the food plus water modular treatment was 39% greater than the water only module. The uptake of ^{14}C-DHP in the mixed model systems, while not statistically different, was lower in both diluters after 24 h than in the modular water only treatment. Depuration data were obtained for the compartmental model only. Significant losses of ^{14}C-DHP occurred in the first hour after transfer to clean medium (Fig. 4), resulting in a 70% loss of activity in the food plus water exposed *Daphnia* and a 64% loss from the water only treatment.

Coefficients of variation (CV) within a treatment, combining replicates from both diluters ($N > 4$), averaged 18% and ranged from 8 to 32%. The variation in results from mixed models was higher (19.5 to 32.0) than for either the water only or food plus water models (8.0 to 21.8 and 8.6 to 20.9, respectively).

Estimates of steady-state BCFs and rate constants (Table 1) show good agreement for the modular approach but less so for mixed model microcosms.

Discussion

Few reports of work addressing the variability of data from microcosms have appeared in the literature. Only studies by Abbott [*20*] and Isensee [*21*] have

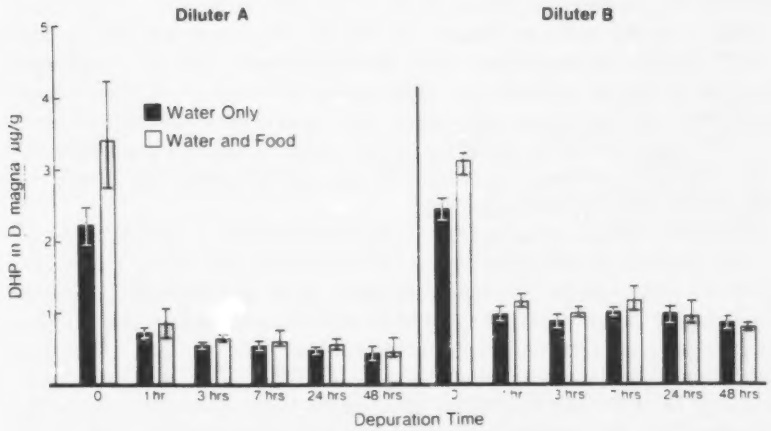

FIG. 4—*Depuration of di-n-hexylphthalate by* D. magna *exposed in modular microcosms in diluters A and B (vertical bars represent range of replicate values).*

TABLE 1—*Measured versus estimated bioconcentration factors for different food chain microcosms.*

Treatment (Source)	Water	Water Plus Food	Mixed Model
Measured BCF	1066	1486	3250
Estimated BCF	999	1414	4655
Estimated K_1	128.4261	128.7096	15.4626
Estimated K_2	0.1286	0.0910	0.0032

specifically set out to test the replicability of microcoms. Isensee [*21*] reported that the multispecies microcosms were about twice as variable as the single-species tests, with coefficients of variation for *D. magna* in mixed model systems extending from 8 to 44 with an average of 22. This is in agreement with our finding which showed CVs of 8 to 21 for the compartmental model and 19 to 32 for the mixed microcosms. In general, the variation that we report is either similar [*20, 21*] or below [*22, 23*] that reported elsewhere.

Brown and Thompson [*8*] in an attempt to understand the discrepancies in the literature on reported bioconcentration (BCF) values for di-2-ethylhexylphthalate found that more complex types of model ecosystems yielded as much as an order of magnitude higher estimates than simpler systems. We also found the mixed species BCF (3250) to be greater than compartmental estimates for water only (1066) or food plus water (1486), but they were within the same order of magnitude. However, these differences were probably caused by length of exposure and not complexity of the model. This may be inferred be-

cause residues on a per weight basis were lower at 72 h in the mixed model than residues after 48 h in the modular model. Isensee [*21*] observed that variability in BCF estimates increases from lower to higher trophic levels but attributed this to an artifact of sampling size, with smaller numbers of individual organisms being represented in samples of fish than for example by *Daphnia*. Hamelink [*18*] suggested that variability in model ecosystem-derived data would increase with time. Although our systems did vary over time no pattern of reduced or increased variability was apparent.

Hamelink [*18*] also suggested that variability was due to changes in chemical concentration in water over time as well as inherent differences in individuals of the same species. Accepting the latter point as a biological principle which we can do very little about, the control of contaminant concentration then becomes a major concern in microcosm tests. The ^{14}C-DHP concentration in our test systems averaged 0.21 μg/L ($\pm$0.025 μg/L standard deviation, $N = 36$). The CV was 8.9, which is less than the range (11 to 31) compiled by Hamelink [*18*]. The reason our systems showed low variability may be due to the relatively constant C_w. The two diluters were true replicates with no statistical differences in measured BCFs between similar treatments from either diluter.

An important consideration of microcosm studies is which system is the most appropriate for estimating chemical concentration in nature. Obviously with the vast number of chemicals which have to be screened for hazard assessment this could be an important economic issue. The type of information needed will determine to some extent the test to be used. If information on specific routes of accumulation is desired, then a modular approach is required. If the aim is to gain a single measure of the total potential residue in a specific trophic level, then either approach could be used.

Care should be taken in using the modular approach suggested by Johnson [*1*] because an equilibrium level may not be attained during the course of the 48-h experiment. A pharmacokinetic approach [*6,18*] to estimate a steady-state BCF might be needed. Tests for estimates of a BCF from modular systems should include animals that were fed even over a short-term experiment, especially when dealing with rapidly metabolizing lower trophic organisms such as *Daphnia*. An inherent design problem in the Isensee and Yockim mixed model was allowing predation on *Daphnia* by fish. Although predation was limited by time (1 h) and passage of *Daphnia* through a screen, we experienced shortages of *Daphnia* for sampling as early as one week into the exposure. This problem may also be exacerbated if the chemical being tested has a negative effect on *Daphnia* reproduction. In our case no data are available on ^{14}C-DHP; however, another phthalate DEHP decreased *Daphnia* reproduction to 40% of the controls, at concentrations as low as 3 μg DEHP/L [*24*].

A review of *Daphnia* nutrition by Buikema et al [*25*] reported work by Lemke and Lampert [*26*] which indicated that unfed *D. pulex* will lose up to 74.4% of their lipids in 48 h, reducing the size of that compartment for accu-

mulation. We found water only estimates of BCF (1066) to be 39% less than those for food plus water (1486). Whether this difference was due to food sources of chemical or higher lipid levels in fed *Daphnia* was not determined. Our attempt to estimate the input from food alone was unsuccessful. Although no tests were performed, this was probably due to a rapid depuration of ^{14}C-DHP by algae upon introduction to unlabeled water and the ability of the *Daphnia* to depurate accumulated ^{14}C-DHP (Fig. 4). The 75 mg of algae fed to the *Daphnia* contained 0.00825 μg ^{14}C-DHP and 638 CPM of ^{14}C-DHP. This activity diluted in 70 L (if all ^{14}C-DHP was depurated by algae) would result in only 0.09 CPM ^{14}C-DHP/mL above background and therefore prevented detection in water.

Conclusions

The residue levels of di-*n*-hexylphthalate in *Daphnia* were one order of magnitude less than previously reported for two species of planktonic marine fish [*11*]. No previous data exist for ^{14}C-DHP residues in other aquatic organisms. Identical designs of relatively simple aquatic food chain microcosms appear to provide reproducible estimates of bioconcentration for ^{14}C-DHP as evidenced by duplicate experiments in separate diluters. Replication of treatments was considered sufficient. Differences in the design of the microcosms and sampling times resulted in up to a three-fold difference in measured BCF for ^{14}C-DHP in *D. magna*. All microcosm designs resulted in measured and estimated BCFs within the same order of magnitude.

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